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THE ROLE OF ESTROGENS AND THE MICROENVIRONMENT IN LYMPHOMA PROGRESSION AND DRUG SENSITIVITY

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THE ROLE OF ESTROGENS AND THE MICROENVIRONMENT IN LYMPHOMA PROGRESSION AND DRUG SENSITIVITY

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To my family
Thanks for your support and for waiting

致家人
感恩你们的支持和等待

ABSTRACT

Lymphomas are a group of cancers which originate from lymphatic cells. More than 90% of the lymphomas belong to the group of Non-Hodgkin lymphoma (NHL), which in itself is a non-homogeneous group consisting of different NHL subtypes. Traditionally, lymphomas are not thought of as hormone-related cancers. This is strange considering that epidemiological studies show a clear gender difference in incidence for most lymphomas with men being more affected and females showing a better prognosis when compared to males. The aim of this thesis was to study molecular mechanisms involved in this gender difference with a focus on sex hormones, particularly estrogens acting through estrogen receptor β (ERβ). Furthermore, since both survival and drug sensitivity of lymphomas can be affected by its microenvironment, we analyzed the impact of stromal cells on ibrutinib sensitivity of mantle cell lymphoma (MCL) and how stromal cell-mediated ibrutinib resistance can be overcome.

Paper I. In this study, gender aspects and estrogen regulation of diffuse large B-cell lymphoma (DLBCL) was studied. When studying DLBCL in a xenograft mouse model, we observed a faster tumor growth in males than in females. Tumor growth was significantly inhibited when mice were treated with the ERβ agonist diarylpropionitrile (DPN). This was the case both for germinal center and activated B-cell like subtype DLBCL. In addition, when analyzing nuclear ERβ1 expression by immunohistochemistry in primary DLBCLs, ERβ1 expression was found in 89% of the cases. No gender difference in ERβ1 expression was found. However, the association between ERβ1 expression and prognosis was complex and shown to be dependent on drug treatment. These results suggest that targeting estrogen signaling via ERβ1 could be a new approach in treating DLBCL. Furthermore, ERβ1 expression might be used as a prognostic marker.

Paper II. In this article, we showed that castration of C57BL6 male mice accelerated tumor growth of lymphoma in C57Bl6 mice engrafted with murine EG7 T cell lymphoma cells. By analyzing the tumor tissue, we observed stronger Ki67 and weaker TUNEL staining in castrated mice, indicating increased proliferation and decreased apoptosis. Treatment of intact male mice with the androgen receptor antagonist Bicalutamide had no effect on lymphoma growth, suggesting that signaling via the androgen receptor is not involved in the enhanced EG7 lymphoma growth following castration. In contrast, accelerated lymphoma growth was observed when mice were treated with aromatase inhibitors (AIs), which inhibit androgen-to-estrogen conversion. Similar effects where seen in mice grafted with human Granta-519 MCL cells or when treated with different AIs. Moreover, dihydrotestosterone, which can't be

converted to estrogens by aromatase, did not have any impact of lymphoma growth in male mice. This further supported an indirect effect of androgens on lymphoma growth, which involves the conversion of endogenous testosterone to estrogens. We conclude that blocking estrogen synthesis may accelerate lymphomas growth, which highlights the protective role estrogens may have on lymphoma progression. Furthermore, our results may raise concern for increased lymphoma risk (at least progression) in breast cancer patients treated with AIs.

Paper III. In this report we showed that MCL tumor growth derived from engrafted human MCL and Burkitt lymphoma (BL) cells was inhibited by treatment with selective agonists of ERβ. Using the selective ERβ agonist DPN, we in tumors derived from Granta-519 MCL cells also demonstrated reduced expression of BAFF and GRB7, two markers of B-cell proliferation and survival. In addition, DPN treatment inhibited the expression of VEGF-C, LYVE-1, CD34 and angiogenin, markers of angiogenesis and lymphangiogenesis. Moreover, by investigating Raji BL cells engrafted to mice, we found that DPN treatment reduced tumor dissemination. Importantly, ERβ protein expression was demonstrated in primary MCL specimens. These results support that lymphomas are under estrogen control and suggest that treatment with ERβ agonists may be a new approach in the treatment of at least some lymphoma subtypes.

Paper IV. By using a MCL-stromal cell co-culture system, we demonstrate that stromal cells protect MCL from ibrutinib-induced apoptosis and support MCL cell regrowth after drug removal by impairing ibrutinib-mediated repression of phosphoinositide-3-kinase (PI3K)/AKT signaling. Importantly, the stromal cell-mediated ibrutinib resistance was overcome by inhibiting AKT activity using the PI3Kα specific inhibitor BYL719. Enhanced pro-apoptotic effect by combined ibrutinib plus BYL719 treatment was also observed in primary MCL cells when co-cultured with stromal cells. Moreover, the combined treatment with ibrutinib and BYL719 enhanced inhibition of MCL tumor growth *in vivo* in xenograft experiments. We found that a direct interaction between MCL cells and stromal cell conferred the ibrutinib resistance and that this involved the adhesion molecule VLA-4. Blocking VLA-4 abrogated the ibrutinib resistance as assayed in a cell apoptosis and a cell regrowth assay. Our results suggest that combined treatment with ibrutinib and a PI3Kα inhibitor, alternatively blocking VLA-4, may be a promising therapeutic strategy to overcome stromal cell-mediated ibrutinib resistance in MCL.

LIST OF SCIENTIFIC PAPERS

I. Hasni MS, Berglund M, Yakimchuk K, **GUAN J**, Linderoth J, Amini RM, Enblad G, Okret S.
Estrogen receptor β1 in diffuse large B-cell lymphoma growth and as a prognostic biomarker
Leuk Lymphoma 58(2):418-427. 2017

II. Talaber G, Yakimchuk K, **GUAN J**, Inzunza J, Okret S
Inhibition of estrogen biosynthesis enhances lymphoma growth in mice
Oncotarget 7(15), 20718-27. 2016

III. Yakimchuk K, Hasni MS, **GUAN J**, Chao MP, Sander B, Okret S.
Inhibition of lymphoma vascularization and dissemination by estrogen receptor β agonists
Blood 123(13): 2054-2061.2014

IV. **GUAN J**, Yakimchuk K, and Okret S
PI3Kα inhibition overcomes stromal cell-mediated resistance to the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

ABC-DLBCL	Activated B cell like diffuse large B-cell lymphoma
AF	Transactivation domain
AR	Androgen receptor
BCR	B-cell receptor
BL	Burkitt lymphoma
BTK	Bruton tyrosine kinase
CLL	Chronic lymphocytic leukemia
CR	Complete response
DBD	DNA binding domain
DHT	Dihydrotestosterone
DPN	Diarylpropionitrile
E2	17β-estradiol
ER	Estrogen receptor
ERE	Estrogen response element
FDA	U.S. Food and Drug Administration
FDC	Follicular dendritic cell
GCB-DLBCL	Germinal center B cell like diffuse large B-cell lymphoma
HL	Hodgkin lymphoma
IKKβ	IκB kinase β
LBD	Ligand-binding domain
MCL	Mantle cell lymphoma
MM	Multiple myeloma
NF-κB	Nuclear factor-κB
NHL	Non-Hodgkin lymphoma
ORR	Overall response rate
PI3K	Phosphoinositide-3 kinase
PMBL	Primary mediastinal B cell lymphoma
TME	Tumor microenvironment

1 BACKGROUND

1.1 LYMPHOMA

Lymphoma is a cancer which derives from cells of the lymphoid system. Lymphoma is a heterogeneous group of cancer. Generally, lymphoma can be classified into Hodgkin lymphoma (HL) and Non-Hodgkin lymphoma (NHL) and they affect both children and adults.[1] NHL is more common than HL and accounts for about 90% of all the lymphomas. Generally, the incidence increases with age, especially for some NHL subtypes like diffuse large B-cell lymphoma (DLBCL) and Mantle cell lymphoma (MCL). Some other lymphomas like HL and NHL Burkitt lymphoma (BL) show a biphasic incidence with one peak appearing in children/adolescents and a second in adults/elderly.[2] An alternative and more up-to-date division of the various lymphomas and more clinically related is the classification based on the tumors growth characteristics into aggressive and indolent NHL, respectively. Follicular lymphoma (FL) and chronic lymphocytic leukemia (CLL) are considered as indolent lymphoma with the characteristic of slow growth but difficult to cure. In contrast, NHL such as DLBCL, BL and MCL are usually considered as aggressive lymphoma with characteristics of fast clinical progression but often more sensitive to intensive therapy, at least in the initial state. However, many of the aggressive lymphomas may relapse. This is e.g. the case with MCL, which is considered incurable. (http://www.cancer.net/cancer-types/lymphoma-non-hodgkin/subtypes). The causes for lymphomas are unknown but some risk factors are high age, male gender, infection (e.g. by Epstein-Barr virus), autoimmune disease, immunosuppression, exposure to some chemicals (e.g. some solvents) and in rare cases heritage.

1.2 NHL

NHL occurs worldwide but is more common in developed countries and is the fifth most common cancer in USA (19 cases per 100 000), while in developing countries like Thailand and China, the reported rates are lower (2-3 cases per 100 000).[3] NHL can be divided into B-cell lymphoma and T-cell lymphoma based on which kind of precursor lymphocyte the lymphoma originates from. There are also some rare forms of NHL such as lymphomas from natural killer cells.[4] Among NHL, B-cell lymphomas make up about 95% of all NHL, and their origin from different stages during the B cell differentiation process are generally well established.[5] The different stages in the B cell maturation takes place mainly in three compartments, the pre-germinal center, germinal center and post-germinal center, respectively, and B-cell lymphomas can develop at any stage during the normal B cell

development. Generally, Mantle cell lymphoma (MCL), Small lymphocytic leukemia (SLL) and Chronic lymphocytic leukemia (CLL) develop in pre-germinal center; Burkitt lymphoma (BL), Germinal center B cell like diffuse large B-cell lymphoma (GCB-DLBCL) and Follicular lymphoma (FL) develop in germinal center while activated B cell like diffuse large B-cell lymphoma (ABC-DLBCL) and Multiple myeloma (MM) develop in post-germinal center (**Figure 1**). Among the B-cell lymphomas, DLBCL is the most common subtype, accounting for 30-40% of the B-cell lymphomas. This is followed by FL 20%, CLL 7%, MCL 6% and BL 2%.[6]

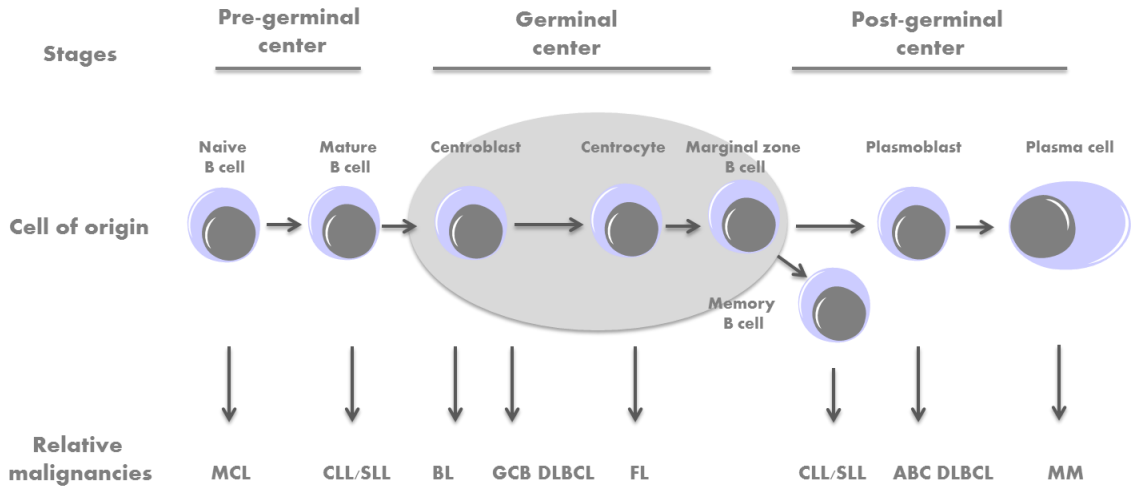

Figure 1: Overview of the stages of B-cell maturation and the association to the different B cell lymphomas [6]

1.3 DLBCL

DLBCL is considered as an aggressive B-cell malignancy because of its fast growth. Another feature of DLBCL is the heterogeneity, with about 40% of DLBCL patients responding to chemotherapy very well, while the rest do not. Gene expression profiling has been introduced to distinguish between different DLBCL subtypes and three major molecularly different forms have been identified. The DLBCLs originating in the germinal center display gene expression patterns similar to germinal center B cell, while the DLBCLs derived in post-germinal center are more like *in vitro* activated B cells.[7] Based on the above features, DLBCL can be divided into GCB-DLBCL and ABC-DLBCL. In clinical trials, patients with GCB-DLBCL have a better prognosis than those with ABC-DLBCL, and recently reported data indicate that a high constitutive NF-κB activity in ABC-DLBCL may contribute to this poorer prognosis.[7-9] In addition, primary mediastinal B cell lymphoma (PMBL), a third subtype of DLBCL has also been identified based on the different gene expression profiling.

PMBL patients are usually younger and have a more favorable outcome compared with the other DLBCL patients.[10]

1.4 MCL

MCL is considered as an incurable B cell malignancy with poor prognosis, and accounts for approximately 6% of all cases of NHL. MCL occurs mainly in elderly population (median age 73.5 years old).[2] MCL usually involves the bone marrow and can be also found in peripheral blood (leukemic spread).[11] MCL express CD5, CD19, CD20, CD22, but lack CD23 expression.[12,13] It is characterized by the t(11;14)(q13;q32) translocation, which results in ectopical Cyclin D1 (CCND1) expression.[14] The t(11;14)(q13;q32) translocation juxtaposes CCND1 at 11q13 to the 14q32, where the immunoglobulin heavy chain complex (IGH) is located. This is considered as the primary event of MCL development. CCND1 overexpression causes a G1/S phase transition, thereby accelerating MCL growth. However, CCND1 overexpression is not sufficient for development of MCL.[15] Apart from the dysregulation of the cell cycle, cell survival signaling pathway activation and change of DNA damage response also contribute to the MCL development.[15,16] After the t(11;14)(q13;q32) translocation in the bone marrow, cells move to the peripheral lymphoid organs like lymph nodes, where most of them locate in the mantle zones without entering into the germinal center.[17] However, recently it has also been found that 15% to 40% of MCLs carry IGHV hyper-mutations, suggesting that these MCLs move into the germinal center. This observation displays a more complex scenario in the classification of different subtypes of MCLs.[18,19] In addition, CCND1 negative MCL cases have also been identified, which showed similar clinical characteristics and gene expression profiling as CCND1 positive MCL. Apart from CCND1, new MCL markers are now being used to distinguish between MCL and other B cell malignancies. Lymphoid enhancer-binding factor 1 is usually negative in MCLs while SOX11 is positive in most MCL cases. It has been reported that *SOX11* aberrant expression contributes to the classical MCL pathogenesis while SOX11 is absent in lymphoid progenitors and mature B cell lymphomas.[20,21]

1.5 GENDER, SEX HORMONES AND NHL RISK

1.5.1 Gender difference of NHL

It is reported that NHL is about 30% to 50% more common in men compared to women.[3,22,23] This includes DLBCL, the most common NHL subtype, which shows a M/F incidence ratio of 1.2 to 1.6:1 in patients of the age of 50 to 85 years.[24,25] Interestingly, among different NHLs, the M/F ratio vary a lot, for instance MCL have a very high M/F incidence ratio (2-

7:1), while the second most common subtype, FL, shows no incidence difference between genders.[11,25] Not only has a gender bias been observed in B-cell malignancies, but also in peripheral T-cell derived lymphomas, with a M/F incidence ratio of 1.8:1.[26] Moreover, from a recent Swedish population based study of DLBCL, male gender was found to be an adverse risk factor for patients less than 52 years of age. For females in the same age group, survival rate was higher, but this difference disappears after menopause.[27] Based on this and other epidemiological data, it is not unlikely that the gender difference in lymphoma incidence could be related to sex hormones, suggesting that lymphoma could be a sex hormone-related cancer.

1.5.2 Sex hormones and NHL risk

In line with the above epidemiological studies, indicating a role of sex hormone in lymphoma development, it has further been reported that reproductive factors and exogenous hormone exposure is associated with a reduced risk of DLBCL in women.[23] Furthermore, pregnancies are significantly related to a decreased NHL risk.[28,29] This could partially be explained by the fact that exposure to reproductive hormones such as estrogen may alter immunity and slower NHL development in women.[23] With respect to post-menopausal hormone therapy, some studies showed that compared with non-users, estrogen therapy causes a decreased NHL risk, while other reports demonstrated an increased risks among long-term users compared with non-users.[30,31] From the above studies, sex hormone-modulated NHL may be complex, and this could partially be explained variation in steroid metabolism, the presence of sex hormone receptors in relevant target cells and an interaction between the endocrine and immune systems.[29] Given the existing complexity in the endocrine system, immune system and the heterogeneous features of NHL, there is an important need to uncover the association between sex hormones and NHL development and progression and to understand the underlying molecular mechanisms.

1.6 ESTROGEN RECEPTOR SIGNALING

1.6.1 Estrogens

Estrogens are C18 steroid hormones derived from cholesterol. Under the cleavage by cytochrome P450 enzymes, different steroids are formed by reducing carbon atoms number from 27 to 18. The last step in the synthesis of estrogen involves aromatization of the A-ring of androgens (testosterone) (**Figure 2**), a process executed by the aromatase enzyme (encoded by gene *CYP19A1*), which is mainly expressed in the ovary and placenta, but also in various extra-gonadal tissues like liver, brain, adipose tissue and skin fibroblasts.[32-36]

Estrogens are present physiologically in the form of 17β-estradiol (E2), estrone (E1) and estriol (E3). E2 is the main physiologically active form and with higher relative binding affinity to the estrogen receptors.[36] With regard to pre-menopausal women, the primary source of estrogen is the granulosa cells in the ovaries, and estrogen production varies cyclically.[37,38] In postmenopausal women, serum estrogen concentration (< 20 pg/ml) is lower than in pre-menopausal women (40-500 pg/ml), and the source is from androgens produced in the adrenal cortex, which undergo conversion to estrogen in extragonadal tissues by aromatase.[37] The primary source of estradiol in male is adipose tissue and testis, where testosterone is converted into estradiol in the process of aromatization.[36,39] Estrogens play important roles both in physiological and pathological processes like cell growth, development and differentiation. The effects of estrogens are found in uterus, breast tissue, central nervous system, bone, immune system and many other organ systems. For instance, the growth of ductal epithelium and connective tissue in human breast tissue is stimulated by estrogens, thus being a risk factor for development of breast cancer.[37]

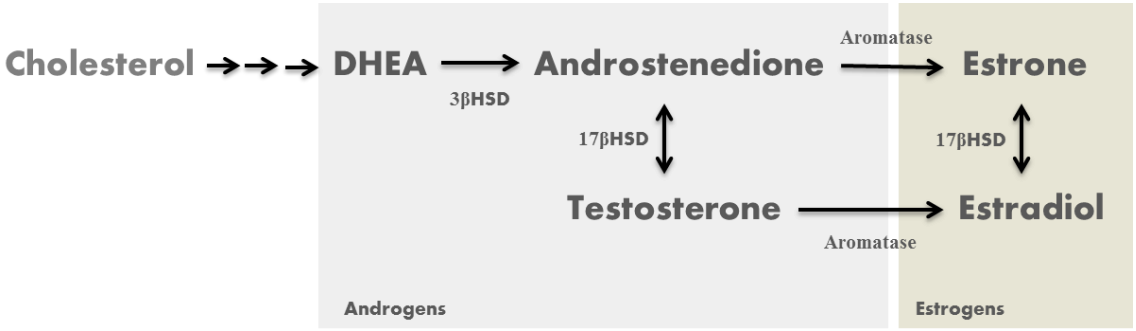

Figure 2: Classical pathway of estrogen biosynthesis from cholesterol.[36]

1.6.2 Estrogen receptors

Estrogens function in their target tissue through two nuclear receptors, estrogen receptor α (ERα) and estrogen receptor β (ERβ), which are the products of two separate genes, *ESR1* and *ESR2*, respectively.[40] The ERs belong to the nuclear receptor family and are widely expressed in many tissues, although ERα and ERβ show a different tissue distribution. ERα expression is mainly found in reproductive tissues, breast, bone, kidney and liver, while ERβ is mainly expressed in the central nervous system, cardiovascular system, male reproductive organs, lung, colon and the immune system.[40-42] In lymphocytes, ERβ has been shown to be highly expressed. Furthermore, in lymphomas, ERβ expression is also very high (**Figure 3**) while ERα level is very low or undetectable.[42,43] The two ERs may display their different functions by recruiting co-regulators (either co-activator or co-repressor) to specific genome targets, thus regulating different gene expression.[44]

Generally, the ER proteins like other nuclear receptors can be divided into six structural regions, which have different functions and are labelled from A to F (**Figure 4**). The A/B region contains a transactivation domain (AF1), which is independent of ligand binding. This region interacts with co-regulators contributing to tissue and gene specific responses. The C region contains the DNA binding domain (DBD), which is required to bind to so called estrogen response elements (EREs) present in the target genes. The D region contains a nuclear localization signal, which helps the transport of ERs into the nucleus. The E/F region harbors the ligand-binding domain (LBD) and a transactivator domain (AF2), where LBD is responsible for ligand binding and AF2 is a ligand-dependent transactivation domain. The AF2 region recruits co-activators or co-repressors thereby contributing to target gene activation or repression.[44] The structure of the two ER subtypes is similar but not identical. When comparing the domains between ERα and ERβ, the DBD domain has 96% conserved residues, the LBD domain shows 58% conserved residues, while the conserved residues in the AF1 domain is only 20%.[44,45] The difference in the AF1 domain most likely explains the differences in target gene activation of ERα and ERβ. The structural differences in the LBD domain have enabled the development of ER subtype specific ligands. When ER ligands bind to the LBD domain of the receptors, a protein conformational change will be introduced, and this is followed by chaperone protein dissociation from the ERs. After that, dimerized ERs translocate from the cytoplasm to the nucleus and bind to the specific EREs in the targeted DNA.[44] After the binding to EREs, the ERs recruit related co-activators or co-repressors to form multi-protein complexes to activate or repress gene transcription respectively.[44] In addition, ligand bond ERs can also interact with DNA indirectly through other transcription factors like AP1 and NF-κB, a mechanism called tethering.[44] Apart from the classical pathway of ligand-dependent ER activation, ER mediated transcription in the absence of ligand binding has also been observed. It is reported that ER activation can be mediated by e.g. epidermal growth factor receptor and insulin-like growth factor 1 receptor in the absence of its ligands by phosphorylation.[46]

Both of the two ER subtypes exist in different isoforms derived from alternative splicing (**Figure 4**). These splice variants are different from the wild type receptors in structure and function, and they are present in both normal and malignant tissues. For example, ERα-36 is a truncated form of wild type ERα. It has been reported to confer endocrine resistance in breast cancer in the presence of wild type ERα. In the case of ERβ, five splice variants have been described (ERβ1-5, ERβ1 being the wild type ERβ). To note is that only the wild type ERβ1 binds ligands efficiently. An important ERβ splice variant is ERβ2 (also called ERβcx),

which in some tissues/cells have been described to be expressed at even higher levels than the wild type ERβ1. The physiological role of ERβ2 is not well established but it has been suggested that it modulates the transcriptional activity of wild-type ERβ (ERβ1) and antagonize wild type ERα through heterodimerization. Moreover, wild type ERα and ERβ form homodimers and may even form a heterodimer. In contrast, ERβ2 and ERβ5 can only form heterodimer with wild type ERs resulting in inhibition of receptor binding to ERE.[44,47]

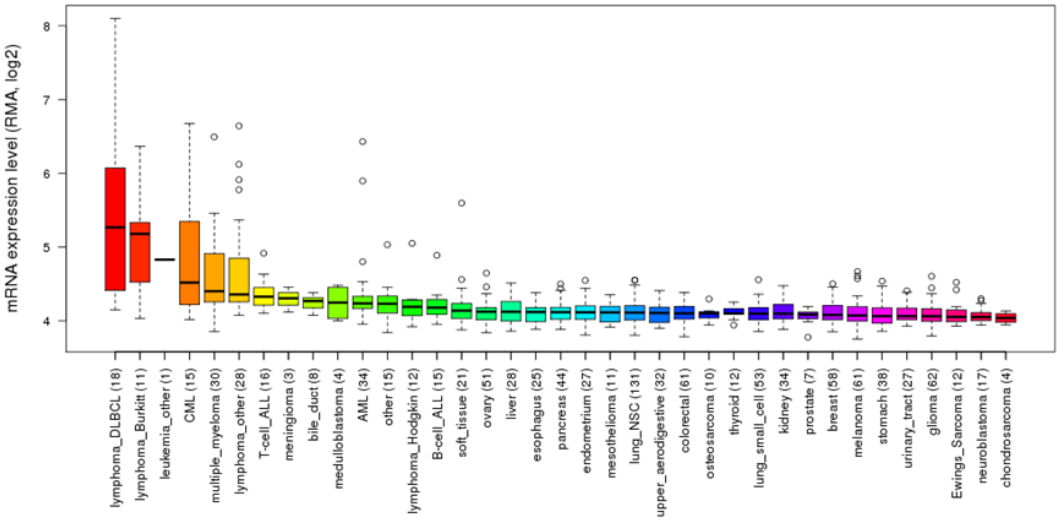

Figure 3: ESR2 is highly expressed in lymphatic malignancies. The data is generated from Cancer Cell Line Encyclopedia database (https://portals.broadinstitute.org/ccle/home).

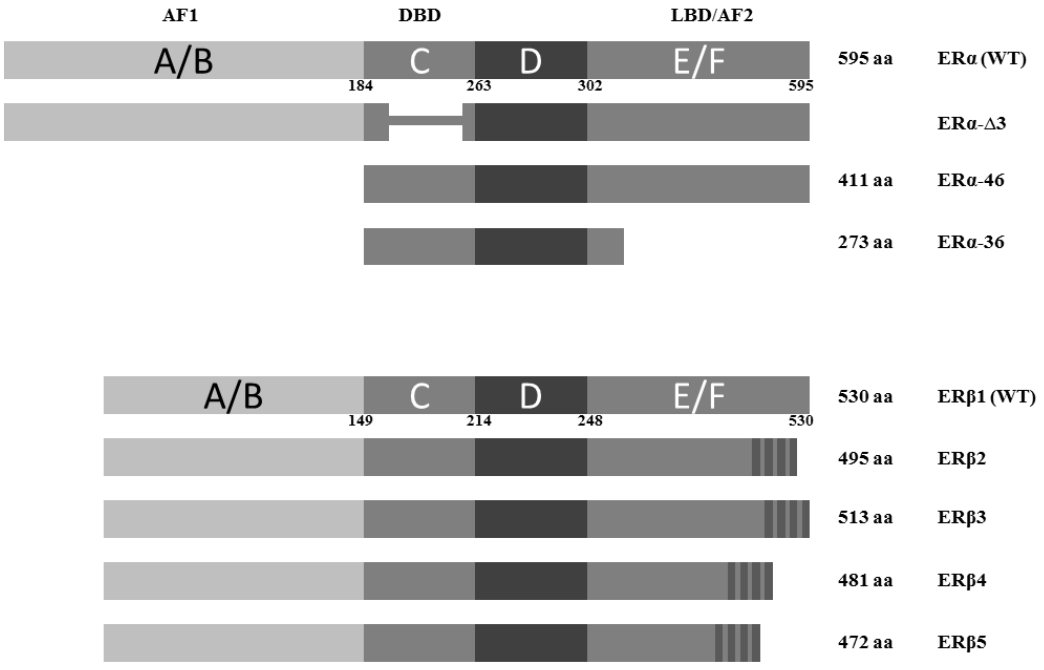

Figure 4: Structural and functional domains of the ERs and splice variants.[44]

1.6.3 Different functions of ERα and ERβ, respectively

Although ERα and ERβ share similar activation mechanism, they usually have different roles in regulating cell proliferation and apoptosis.[48] For example, ERα signaling has been shown to promote cell proliferation while ERβ seems to inhibit proliferation.[49] Similarly, it has been reported that ERα positively regulates nuclear transcription factor MYC, while ERβ does the opposite.[50] Interestingly, when ERβ was introduced into ERα positive breast cancer cells *in vitro*, repression of cell proliferation was observed.[44] The different responses to ERα and ERβ, is likely explained by differences in the protein structures, particularly of AF1 domain, which is involved in recruitment of co-regulator proteins. ERα and ERβ share only 20% amino acid similarity in their AF1 domain.[44]

1.6.4 Estrogen receptor β activation and tumor inhibition

Although there is a controversy about the normal role of ERβ *in vivo* as derived from *Esr2*-knockout mice generated in different labs, an ERβ tumor suppressive function is more commonly accepted considering its clear anti-proliferative effect in *in vitro* and *in vivo* cancer models. For example, in the prostate epithelium of Esr2-knockout mice, epithelial hyperplasia has been observed. In line with the above results, prostate cancer cell proliferation, migration and invasion is inhibited by ERβ overexpression.[51,52] Furthermore, DNA methylation of the ERβ promoter region, thereby reduced expression, is found in more aggressive prostate tumors.[53] Moreover, in squamous cell carcinoma mouse model, ERβ overexpression or ERβ agonist treatment increased the expression of NOTCH1, inhibiting the growth of tumor and promoted tumor differentiation both *in vitro* and *in vivo*.[54] Furthermore, ERβ selective agonist KB9520 not only showed an anti-proliferative effect on malignant pleural mesothelioma cells, but also sensitized the cells to cisplatin induced pro-apoptotic effect.[55]

Although ERβ seems to inhibit tumor growth of several cancer forms, its mechanism of action is still not clear. ERβ has been reported to inhibit tumor growth in G1 phase by regulating cell cycle checkpoint related factors like CCND1, p21, MYC, FOXO1 and p53.[56,57] Recently, ERβ was found to reduce cell survival by regulating RAS signaling pathway in Non-small cell lung cancer.[58] In prostate cancer cells, researchers also found that ERβ could decrease phosphorylation of AKT (Th308), increase the expression of FOXO3a and PUMA which was followed by apoptosis triggered in a ligand (5α-androstane-3β,17β-diol) dependent way.[59] This suggests that activation of ERβ by ERβ agonist could be a novel strategy to kill prostate cancer cells. In line with the above observation of ERβ anti-proliferative effects in cancer cells, we have found in our experiments that activation of ERβ

by its selective agonists DPN or KB9520 can repress murine lymphoma growth *in vivo* which involves both inhibition of proliferation and increased apoptosis.[42]

1.7 B CELL RECEPTOR (BCR) SIGNALING IN B CELL LYMPHOMAS

1.7.1 BCR signaling

BCR signaling is essential for the proliferation of both normal B cells and B cell malignancies (**Figure 5**). This involves increased levels of phosphorylated (active) Bruton tyrosine kinase (BTK) and SYK, two downstream signaling components of the BCR pathway,[60-63] which in turn results in high nuclear factor-kB (NF-κB) activity through the canonical NF-κB signaling pathway. Increased NF-κB signaling has been shown to play a central role in e.g. the pathogenesis of MCL and ABC-DLBCL. In addition, BCR signaling induces activation of Phosphoinositide-3 kinases (PI3K)/AKT, RAS, MAPK and nuclear factor of activated T cell pathway (NF-AT), which all contribute to cell survival, proliferation, adhesion and migration.[64,65]

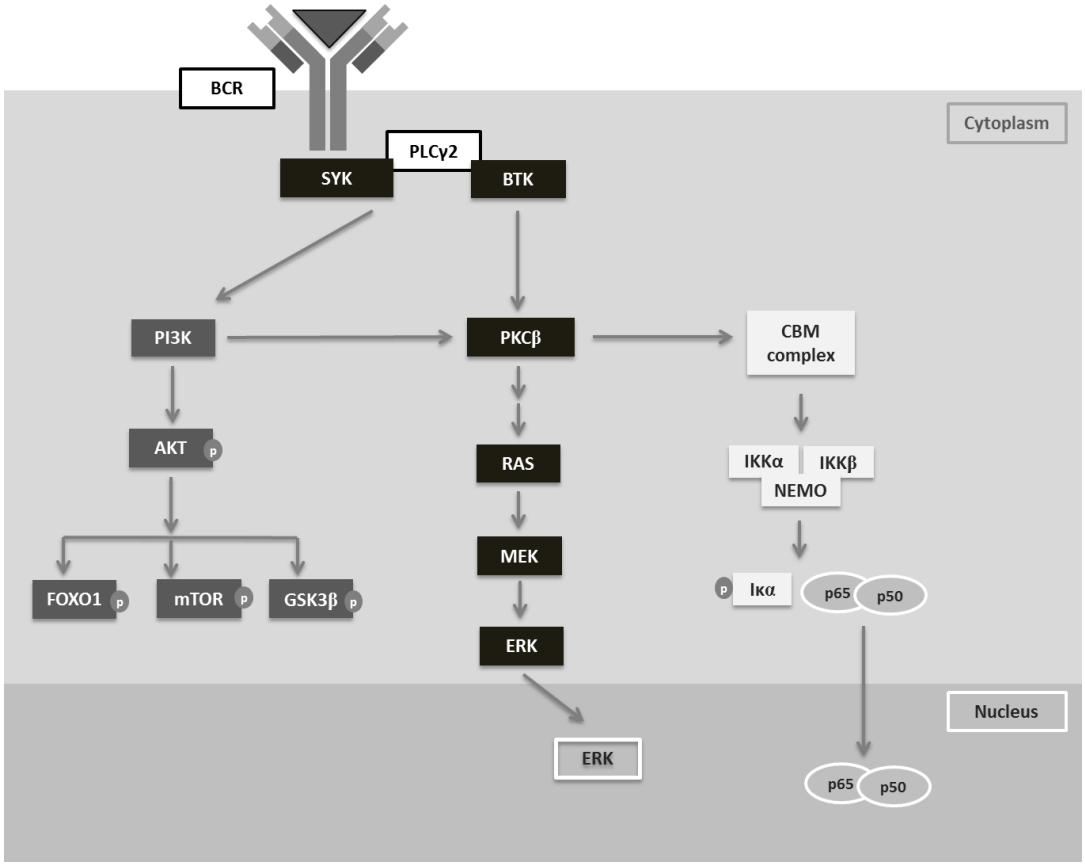

Figure 5: B cell receptor signaling.[60]

1.7.2 NF-κB signaling

NF-κB signaling is a very well-studied pathway in normal and cancer cells. The NF-κB proteins are critical regulators of important physiological processes like immunity, inflammation, proliferation and apoptosis. They have also been suggested to play a survival role in many forms of tumors due to constitutive activation. Constitutive NF-κB signaling also plays an important role in human B-cell malignancies. NF-κB activation occurs downstream of numerous receptors like the BCR, CD40, Toll like receptor 4 and B cell activating factor (BAFF) receptor.[66] NF-κB signaling can be subdivided into a classical pathway and an alternative pathway, which have different roles in cell physiology and pathology. In the classical NF-κB pathway, receptor activation leads to phosphorylation of the IκB kinase β (IKKβ) subunit in the IKK complex (three subunits: IKKα, IKKβ and NEMO/IKKγ), where after IKKβ phosphorylates IκBα, the inhibitor of NF-κB dimerization (p50/p65 and p50/c-Rel). Phosphorylation of IκBα leads to a proteasome-mediated degradation of IκBα allowing NF-κB dimers to translocate to the nucleus, bind to DNA and activate transcription of target genes.[66] In the cases when the alternative NF-κB pathway is activated, the IKKα subunit is phosphorylated by the kinase NIK. IKKα phosphorylates p100, leading to its cleavage into the p52 subunit, which pairs with another subunit, RelB, where after the p52/RelB dimer translocates to the nucleus.[67] Both of these two NF-κB signaling pathways play important roles in the survival and proliferation of normal B-cell development and B-cell malignancies.

With respect to MCLs pathology, it has been reported that in both MCL patient samples and MCL cell lines, NF-κB is constitutively activated and plays an important role in regulating MCLs survival and growth. In B cells, the classical NF-κB pathway is activated by protein kinase Cβ, which is the target of diacylglycerol, released upon BTK activation. By activating the classical NF-κB pathway, BTK plays an essential role in BCR signaling of normal B cell proliferation as well as in B cell malignancies including MCL.[68,69] Interestingly, researchers have found that some MCL lines rely on chronic BCR classical NF-κB signaling and are highly sensitive to the PKC inhibitor sotrastaurin, while other MCL cell lines which are dependent on the alternative NF-κB pathway for survival, are resistant to this drug.[70] The above finding suggests that different B-cell lymphoma subtypes may rely on classical and alternative NF-κB signaling pathways, respectively.

1.7.3 PI3K/AKT signaling

PI3K belongs to the family of lipid kinases translating extracellular signals into intracellular signals that regulate many physiological functions and cellular processes.[71-73] Aberrant PI3K activation is seen in cancers, and it is considered to be part of the MCL pathogenesis.[74] PI3K activates its downstream target serine-threonine kinase AKT, which plays an important role in promoting MCL survival and progression by stabilizing *CCND1* mRNA and by preventing CCND1 nuclear export.[75,76] The family of PI3K consists of four families (class I, II, III, and IV), in which class I PI3K is most well studied.[77] Class IA PI3K transduces signals from receptors tyrosine kinases leading to cell survival and growth.[71] The PI3K class IA consists of one of three catalytic subunit isoforms, p110α (encoded by *PIK3CA*), p110β (encoded by *PIK3CB*), and p110δ (encoded by *PIK3CD*) which dimerizes with p85 regulatory subunit.[76,78] It has been reported that p110δ is highly expressed in MCL, contributing to BCR-induced PI3K activation, while p110α expression level in MCL varies greatly, playing a role in maintaining constitutive PI3K activation.[76] Moreover, some observations have demonstrated that *PIK3CA* gene amplification contributes to the MCL pathogenesis and those MCL patients who relapse express more p110α compared with chemotherapy sensitive patients.[76,79]

1.8 THE TUMOR MICROENVIRONMENT (TME) IN B CELL MALIGNANCIES

TME with its non-malignant cells (e.g macrophages and T cells) and stromal components has a major influence on tumor cells when it comes to tumor proliferation, survival, dissemination and resistance to therapy. This is also the case for NHL.[80] Evidence for TME involvement in lymphoma development includes experiments demonstrating a correlation between the presence of e.g. macrophages or T cells with clinical outcome.[81-83] Furthermore, recent results suggest the interaction between the TME and lymphoma cells to be bidirectional with the lymphoma cells secreting cytokines which in turn attract non-malignant cells like macrophages, immune- and stroma cells that influence the tumor cells.[80] Extrinsic stimulation by the TME through the BCR plays a central role in proliferation and survival of normal and malignant B cells.[84] This highlights the importance of the TME in B cell lymphomas.

Lymphomas can derive from developing lymphocytes at different developmental stages. For B-cell lymphomas, their development usually happens in lymphoid structures in which normal T-cell, other cells or stroma may interact with the tumor cells, stimulating lymphoma cell survival.[85] The B-cell lymphoma TME contains stromal cells, blood vessels, immune cells and extracellular matrix. When normal lymphoid tissue is infiltrated by lymphoma cells,

a mutual interaction between the lymphoma cells and the microenvironment occurs which support lymphoma cell survival or dissemination.[80]

Both direct cell-to-cell contacts and soluble factors of the TME may contribute to the survival signal. The MCL adhesion molecule very late antigen-4 (VLA-4) has been shown to interact with extracellular matrix proteins such as fibronectin or vascular cell adhesion molecule 1 expressed on stromal cells, followed by triggering of survival signals.[86] In addition, soluble factors secreted by the TME also promote tumor cell survival and proliferation. Paracrine interleukin-6 (IL6) can prevent MCL from spontaneous apoptosis and blocking the IL6 receptor gp80 or neutralizing IL6 can inhibit cell growth and induce cell apoptosis. Moreover, IL6 has been shown to activate the Jak2/STAT3 and PI3K/Akt pathways in MCL.[87] Another study showed that IL4 secreted by T Follicular Helper cell can stimulate progression of FL by activating the STAT6 signaling pathway in a paracrine manner.[88] Furthermore, stromal cells may also secrete soluble factors like BAFF to protect e.g. DLBCL and MCL from apoptosis.[89]

1.9 LYMPHOMA MICROENVIRONMENT AND DRUG RESISTANCE

Beyond the experimental evidence for a role of the TME for lymphoma growth, survival and dissemination, there are more and more clinical or pre-clinical evidence demonstrating that the interaction between lymphoma tumor cells and its microenvironment plays an important role in altering the sensitivity of the lymphoma cells to chemotherapy.[80] For example, bone marrow stromal cells have been shown to protect NHL cells from mitoxantrone treatment-induced cell apoptosis, which occurs through NF-κB-mediated upregulation of anti-apoptotic proteins.[90] Lymphoma cell adhesion to follicular dendritic cells (FDCs) mediates cytotoxic drug resistance due to the induction of miR-181a and a reduced level of the pro-apoptotic protein BIM.[91] Furthermore, direct FDC and lymphoma interaction has been shown to increase the activity of MYC by down-regulating miR-548m expression level and to enhance HDAC6 expression leading to lymphoma survival following treatment by cytotoxic drugs.[92] Some studies partially explain the drug resistance mechanism by stating that lymphoma cell adhesion to stromal cells causes cell cycle arrest, which will prevent the cytotoxic effects of chemotherapy.[93]

It is known that MCL as a kind of incurable NHL with high relapse rate and poor prognosis, in advanced stages often home to the bone marrow compartment, from where the cells will obtain survival signals, making the tumor cells more prone to resist drug-induced effects.[50] It has also been shown that mesenchymal stromal cells protect MCL cells from doxorubicin,

fludarabine or bortezomib-induced apoptosis by inducing secretion of BAFF and activation of canonical and alternative NF-κB signaling.[94] IL6 has been reported to derive from cells of the TME or the MCL cells themselves and plays a central role in MCL cell survival and drug (bortezomide) resistance.[87] Taken together, this shows that the TME-MCL interaction contributes to drug resistance *in vivo*, which in turn underlies the cause for minimal residual disease and relapse.

1.10 IBRUTINIB TREATMENT AND RESISTANCE IN MCL

1.10.1 Ibrutinib effect on MCL

Targeting BCR signaling using BTK inhibitors has been shown as a promising therapeutic strategy in clinical studies of some lymphomas. In a recent Phase II trial of MCL, the BTK inhibitor ibrutinib showed an encouraging effect. In MCLs patient, an overall response rate of 68% was observed, 21% of MCL patients achieved a complete response while 47% achieved a partial response. Based on these promising results as well as limited side effects by ibrutinib, the U.S. Food and Drug Administration (FDA) did approve ibrutinib in 2013 for the treatment of MCL patients who have received and failed at least one prior therapy, and in 2014, FDA has also approved ibrutinib for treating CLL patients and later for Waldenström's macroglobulinemia. Trials for treatment of additional lymphomas with ibrutinib, is ongoing.

1.10.2 Ibrutinib resistance in MCL

A follow-up clinical study by Wang and coworkers,[95] in which the BTK inhibitor ibrutinib was evaluated, showed an overall response rate in 67% (complete response in 23%) of 111 MCL cases which previously had shown primary or developed resistance to treatment by other drugs. The remaining one-third of the MCL patients was refractory to ibrutinib treatment. In a study by another group involving 114 MCL subjects with therapy resistance to other drugs, a complete or partial ibrutinib response rate of 50% was seen.[96] Primary resistance to ibrutinib in non-responsive MCL has been explained by constitutive activation of the alternative NF-κB signaling pathway not targeted by BCR signaling inhibitors like ibrutinib, which only hampers the canonical NF-κB signaling pathway.[70] In this study, it was reported that 16% of the MCL patients had a deregulated alternative NF-κB signaling pathway as a result of inactivating mutations in TRAF2 or BIRC3, two genes that are involved in suppressing the alternative NF-κB signaling.[70] Moreover, next-generation sequencing has revealed additional somatic mutations that may contribute to primary drug resistance by BCR signaling inhibitors e.g. NOTCH1 or CARD11.[97-99]

In addition to primary resistance, acquired (secondary) resistance may develop, which is not an uncommon phenomenon in cancer treatment, also with modern targeted drugs. Although the initial response of MCL to treatment often is positive, still most patients eventually relapse. Though 50-67% of MCL patients treated with ibrutinib showed a complete or partial response upon start of ibrutinib treatment (see above), the median duration of response was limited to 3.5-17.5 months, where after drug resistance developed.[95,96] In some, but in a very limited number of the cases, could the ibrutinib resistance in relapsing MCL patients be attributed to an acquired mutation in the BTK (C481S) not present before the start of the ibrutinib treatment.[100,101] The C→S mutation of amino acid residue 481 in the active site of BTK disrupts binding of ibrutinib to BTK, explaining the acquired resistance to ibrutinib. Acquired resistance to ibrutinib still develops in patients with non-mutated BTK post-ibrutinib treatment relatively rapidly. As demonstrated by Martin et al,[96] only 1 out 8 patients with non-mutated BTK post ibrutinib treatment experienced durable response >1 year.

2 AIMS OF THE STUDY

For most subtypes of lymphomas, the risk of developing these malignancies is higher in males compared to females. Furthermore, prognosis is usually poorer in males *vs.* females. Additionally, epidemiological data have suggested that sex hormones, particularly estrogens, may play a role in this gender difference in lymphoma development. Despite this, lymphomas are not considered as sex hormone-regulated malignancies and the molecular mechanisms for this hormone regulation is lacking. Based on this and some previous studies in the group on a murine lymphoma, the investigation of this thesis aimed to answer the following questions:

1. What roles do estrogens and androgens have in regulating lymphoma progression?
2. What are the mechanisms for sex hormone effects on lymphoma progression and which receptors are involved?
3. Can selective estrogen receptor agonists be used to diminish lymphoma progression?
4. How does ERβ expression look like in clinical lymphoma samples and can ERβ act as a prognostic marker?

MCL is known to be an aggressive NHL with poor prognosis, although initial treatment results often are positive, development of treatment resistance and relapse most often occurs, also following treatment with more modern targeted drugs like ibrutinib. Thus, there is a great need for alternative approaches in the treatment of aggressive NHL. In order to uncover the mechanism and overcome ibrutinib resistance, we focused on the TME, as the TME has been shown to protect tumor cells from cytotoxic effects induced by conventional chemotherapies. Thus, except analyzing if MCL progression can be impaired by treatment with selective ERβ agonists (see above), we also wanted to investigate if sensitivity of MCL to ibrutinib could be potentiated

5. By analyzing the impact and mechanisms of stromal cells on the sensitivity of MCL cells to ibrutinib and to identify ways to overcome stromal cell-mediated ibrutinib resistance.

3 RESULTS AND DISCUSSION

3.1 ESTROGEN EFFECTS ON LYMPHOMA PROGRESSION (PAPER I-III)

3.1.1 Systemic estrogens play a protective role in lymphoma progression (Paper I and Paper II)

Epidemiological studies have demonstrated a higher incidence and poorer prognosis of most lymphoma subtypes in males compared to females. Furthermore, epidemiological data have shown that lymphoma development may be under the control of sex hormones, particularly estrogens. Despite this, lymphomas are not considered as hormone-related malignancies. Previous studies in the group had shown that an allografted murine T cell lymphoma grew faster in male mice as compared to female mice, a difference that was abolished following ovariectomy.[42] This suggested a role for estrogens in the control of progression of this lymphoma. To investigate if there was a gender difference of tumor growth also for human lymphomas, human DLBCL cells were grafted to immunocompromised NSG mice. As can be seen in **Paper I Fig.1,** tumors derived from the human ABC-DLBCL lymphoma grew slower in female *vs.* male mice, demonstrating that there is a gender difference in tumor growth both for murine and human, as well as for B and T cell tumors. Further support for this was gained by investigating the role of male sex hormones in the development of lymphomas. After castration, male mice were grafted with the murine T-cell lymphoma EG7 and we found that the castrated mice showed faster lymphoma growth compared to sham-operated mice. However, since androgens can be converted to estrogens by the enzyme aromatase (expressed by the *CYP19A1* gene), we wanted to distinguish whether the effect was a direct androgen effect (via the androgen receptor (AR)) or occurred indirectly by androgen conversion to estrogens. This was done by administrating the aromatase inhibitor (AI) Letrozole, which prevents androgen to estrogen conversion or the AR antagonist Bicalutamide to intact male mice which had been grafted with EG7 lymphoma cells. We observed that Letrozole administration resulted in a faster tumor growth while Bicalutamide treatment did not show any effect on tumor growth compared to vehicle treated mice. These results showed that the effect was not mediated by a direct androgen (via AR) effect but rather an indirect effect via ER by inhibiting androgen conversion to estrogens. This conclusion was supported by the lack of effect on lymphoma growth by dihydrotestosterone (DHT), a potent AR agonist but which can't undergo aromatization to an estrogen. The same stimulation of lymphoma growth by various AI's was seen when analyzing human Granta-519 MCL xenografts in immunocompromised NGS mice. These results showed that the lymphoma growth promoting effect also occurs

with human lymphomas and that both T and B cell lymphomas are affected. Since the AI's enhanced lymphoma growth in immunodeficient mice, it also showed that the effect is independent of an intact adaptive immune system (**Paper II**). The above results highlight the important role of estrogens in protecting against lymphoma progression and raise concern whether women with breast cancer treated with AI may have an increased risk for lymphoma development (at least progression if lymphoma initiation has occurred). Taking the above experimental data from our group together with the epidemiological data strongly support that lymphoma development, particularly progression, is under sex hormone control. Thus, lymphomas should be considered as hormone-regulated cancers.

3.1.2 ERβ activation by its agonists inhibit lymphoma growth (Paper I and Paper III)

We have previously shown that murine T-cell lymphoma (EG7) cells express ERβ (but very little or no ERα) and that EG7 lymphoma growth can be repressed following ERβ activation in an allograft mouse model.[42] Whether this repression of tumor growth *in vivo* was a direct effect on the tumor cells or also involved indirect effects via ERβ activation of cells of the TME or other tissues was not determined. To study this, we treated ERβ-deficient (BERKO) mice and wild-type mice grafted with ERβ positive EG7 lymphoma cells with the ERβ selective agonist DPN and found that DPN inhibited ERβ positive EG7 lymphoma tumor growth similarly in both wild-type and ERβ-deficient mice. This result suggests that ERβ agonists exert a direct effect on the EG7 cells and not indirectly through the TME or other tissues. However, caution with such a conclusion should be taken, since it has been demonstrated that the BERKO ERβ-deficient mice may express truncated versions of ERβ which still may exert some effects.

The ability of selective ERβ agonists to suppress tumor growth was also seen in mice grafted with human B-cell lymphomas e.g. Granta-519 MCL cells and DLBCL cells, which express ERβ mRNA and protein, while ERα levels are very low, (**Paper I, Fig. 2; Paper III, Fig. 2**). In the latter case, impaired tumor growth by the ERβ selective agonist DPN was seen both in mice grafted with GCB-DLBCL (SU.DHL4) and ABC-DLBCL (U2932) cells demonstrating that inhibition of DLBCL growth by ERβ is independent of whether NF-κB activity is high (U2932 ABC-DLBCL) or low (SU.DHL4 GC-DLBCL) (**Paper I, Fig. 3**). Impaired lymphoma growth following ERβ agonist treatment was also seen in tumors derived from Raji BL cells (**Paper III**). The ability of selective ERβ agonists, particularly KB9520 which has a 700-fold selectivity for ERβ activation over ERα

activation (**Paper III**) to suppress tumor growth, supports that the main contributor to the tumor inhibiting effect is ERβ. Importantly, the ERα selective agonist PPT had no effect on tumor growth, excluding the possibility of an ERα-mediated effect. The ER antagonist ICI182.780 could partially block the tumor suppressing effect of DPN, further supporting engagement of ER signaling in the response. Interestingly however, ICI182.780 seems by itself to act as a partial agonist on the ERβ as defined by its ability by itself to partially block tumor growth (**Paper III**). Another interesting observation was that E2, which binds to ERβ with the same affinity as it binds to ERα, did not repress MCL tumor growth (**Paper III**) and to a very limited extend repressed EG7 lymphoma growth[42]. The cause for E2's inability to repress lymphoma growth despite its ability to activate ERβ (at least is other cells) is unclear, but may be explained by an inability of the E2-ERβ complex to recruit proper co-regulators in the lymphoma cells.

The target genes of ERβ in repressing lymphoma growth are still unclear. However, we identified two genes in the Granta-519 MCL tumors that could participate in this process, namely the tumor proliferation gene GRB7 and the B cell survival gene, BAFF. Expression of the genes were down-regulated by the DPN treatment. (**Paper III, Figs. 2, S3 and S4**). Collectively, ERβ activation inhibits the growth of a number of NHLs with different origins and molecular characteristics. This suggests that the mechanisms for the tumor repressing effect by ERβ in various lymphomas should be more general and uncovering this mechanism would be both theoretically important (explain the gender difference in prognosis and possibly incidence) and clinically significant as a new treatment option.

3.1.3 Lymphoma vascularization and dissemination are inhibited by ERβ agonist treatment (Paper III)

By analyzing tumor material derived from Granta-519 MCL cell engrafted mice, we observed downregulated expression of different vascularization related genes following DPN treatment. This included down-regulated mRNA expression of LYVE-1 (a marker of lymphoendothelial cells), CD34 (partially a marker for blood vessels) and angiogenin. The down-regulated LYVE-1 mRNA expression was associated to a reduced number and size of lymph vessels as determined by immunohistochemistry. The reduced vascularization correlated to a down-regulation of vascular endothelial growth factor C mRNA expression, supporting that ERβ activation inhibits tumor lymphangiogenesis and angiogenesis. Because tumor vascularization is considered as an important factor for tumor progression, including tumor growth and tumor dissemination, we also examined the effect of ERβ agonist treatment on lymphoma dissemination by administrating DPN to mice engrafted

with Raji BL cells, which previously have been reported to display significant liver dissemination. It was observed that ERβ activation reduced the dissemination of Raji BL tumors in the xenograft mouse model as indicated by both a decreased number as well as a reduced size of the metastatic tumors. (**Paper III, Figs. 3-5; Figs. S6-8**)

3.1.4 ERβ expression in primary lymphoma samples and its potential role as a prognostic biomarker (Paper I and Paper III)

To identify if ERβ is expressed also in primary lymphoma samples and not only in lymphoma cell lines, we identified ERβ1 expression in both primary MCL (**Paper III**) and DLBCL (**Paper I**) samples, suggesting that ERβ can be considered as a novel therapeutic target. We focused on ERβ1 expression since this is the wild-type ERβ variant able to efficiently bind a ligand (see Background). In the case of DLBCL, ERβ1 expression was found in 89% of the DLBCL samples, although at various levels. After comparing nucleus ERβ1 protein expression levels to clinical data, we have found a significant correlation between negative/low ERβ1 levels and extra-nodal localization, which may reflect that disseminated lymphoma cells represent a dedifferentiated cancer stage that may be insensitive to estrogen regulation (**Paper I**). No correlation to gender, DLBCL subtype (GC *vs.* non GC-DLBCL), pre- *vs.* postmenopausal age or other clinical characteristics was observed. When analyzing the correlation between ERβ1 expression levels with patient survival, we found that in the CHOP-treated patient cohort, intermediate/high nuclear ERβ1 expression correlated to a favorable prognosis of both overall survival and progression-free survival. This is in line with observations in some other tumors.[102] However, in the Rituximab-CHOP-treated patient cohort, the inverse correlation was observed with low ERβ1 expression correlating to better progression-free survival. The reason for this inverse relationship between ERβ1 expression and survival in the patient cohorts treated with CHOP with or without Rituximab is not clear. However, it is not due to unequal distribution of base line characteristics since they did not differ significantly between patients with high or low ERβ1 expression level. Furthermore, statistical significance was maintained when compared with other prognostic factors in the multivariate analysis. To note is that a similar phenomenon has been shown for other lymphomas where the prognostic impact of a biomarker is impaired or inversed after adding Rituximab to a drug treatment scheme.[103-105] Independent of this, the results suggest that targeting ERβ1 by its agonists is a promising therapeutic way for treating DLBCL, and that ERβ1 expression can be used as a prognostic marker.

3.2 STROMAL CELL EFFECTS ON IBRUTINIB SENSITIVITY OF MCL (PAPER IV)

3.2.1 Stromal cell-mediated ibrutinib resistance through regulation of AKT activity in MCL

Ibrutinib administration to mice grafted with Mino MCL cells showed only a transient inhibitory effect on tumor growth, as with time a resistance to ibrutinib developed. In order to investigate whether this may be due to an impact of stromal cells on the sensitivity of MCL to ibrutinib, MCL cells were co-cultured with murine MS-5 stromal cells and treated with ibrutinib. Co-culturing the MCL cells with MS-5 stromal cells resulted in reduced inhibition of MCL cell proliferation by ibrutinib compared to when MCL cells were grown alone. Stromal cells also reduced sensitivity of Mino and Rec-1 (another MCL cell line) cells to ibrutinib-induced apoptosis. The above results demonstrate that stromal cells impair MCL cell sensitivity to ibrutinib. Importantly, if Mino cells which had become resistant to ibrutinib-induced cell apoptosis in the presence of stromal cells were transferred into new wells without stromal cells and retreated with ibrutinib, they regained sensitivity to ibrutinib-induced apoptosis. This confirmed that stromal cells, and not a tumor cell autonomous mechanism, conferred the resistance. Furthermore, the inability of stromal cell conditioned media to prevent ibrutinib-induced cell apoptosis demonstrated that a direct stromal cell-MCL interaction is the main trigger for the resistance. These results emphasize that apart from acquired BTK mutation for development of ibrutinib resistance, stromal cells have an important role in providing a protective niche to make MCL cells resist ibrutinib-induced effects.

We then investigated whether the stromal cells will affect the restart of proliferation when ibrutinib is removed. Rec-1 MCL cells co-cultured with MS-5 stromal cells were treated with ibrutinib for 10 days. After 10 days of ibrutinib treatment, proliferation of Rec-1 cells was largely absent. However, 6 days after the ibrutinib was removed, a substantial recovery of proliferating Rec-1 cells was observed. When no stromal cells were present during a 2 day ibrutinib treatment, no recurrence of proliferating Rec-1 cells was seen 6 days after ibrutinib was removed. To further support that the stromal cells impaired sensitivity to the anti-proliferative effect of ibrutinib, the response of Rec-1 cells adhering to the stromal cells and the cells growing in suspension (non-adhering) were analyzed separately. The reappearance of proliferating cells after ibrutinib removal was shown to originate from the stromal adherent lymphoma cells and not from the lymphoma cells growing in suspension (non-adherent cells). This further supports that it is the stromal cells that confer ibrutinib resistance to the MCL, allowing tumor cells to regrow after ibrutinib is removed. This also

supports that a direct physical contact between stromal cells and lymphoma cells is required to confer the ibrutinib resistance.

Ibrutinib regulates both the BCR-NF-κB and PI3K/AKT signaling pathways. Our initial results indicated that the inhibitory effect of ibrutinib on the NF-kB signaling pathway in MCL cells remained intact in the presence of MS-5 stromal cells. We therefore focused our investigation on the effect of stromal cells on the PI3K/AKT signaling pathway. In the absence of stromal cells, ibrutinib strongly reduced pAKT (Ser473) levels, while co-culturing the lymphoma cells with MS-5 stromal cells impaired the ibrutinib-mediated down-regulation of pAKT. In the cases when Mino, Rec-1 and Jeko-1 cells were co-cultured with human FDCs, increased levels of pAKT was seen. These results suggest that stromal cell interaction with MCL cells increase pAKT levels or impair ibrutinib-mediated down-regulation of AKT signaling in the MCL cells.

3.2.2 Overcoming stromal cell-mediated ibrutinib resistance

In order to test if the increased pAKT activity in MCL cells conferred the stromal cell-mediated ibrutinib resistance, we screened inhibitors of PI3Ks for their ability to restore MCL cell sensitivity to ibrutinib-induced apoptosis in the presence of MS-5 stromal cells. Mino cells were treated with different PI3K inhibitors alone or in combination with ibrutinib. Combined treatment between ibrutinib and BYL719, a PI3Kα inhibitor, resulted in a strong apoptotic response with a clear synergistic effect compared with single drug treatment. The pro-apoptotic effect of ibrutinib+BYL719 was much more pronounced than the effect seen when ibrutinib was combined with other drugs, including inhibitors of other PI3K family members. Treatment with either ibrutinib or BYL719 resulted in reduced levels of pAKT in the lymphoma cells. Combined treatment with ibrutinib and BYL719 resulted in at least an additive inhibitory effect on pAKT levels. Importantly, a synergistic effect of BYL719 and ibrutinib on the induction of apoptosis in the presence of stromal cells was also seen in primary MCL cells. The above results further confirm that AKT activity confers ibrutinib resistance. We also observed that ibrutinib and BYL719 inhibited cell proliferation separately, and no additive effect was seen when combined. However, in the case when MCL (Mino)-stromal cells (MS-5) co-cultures were initially treated with ibrutinib before it was removed, restart of Mino cell proliferation in the presence of MS-5 stromal cells occurred. In contrast, impaired restart of Mino cell proliferation was seen in culture plates that were treated initially with both ibrutinib and BYL719 before drug removal. This correlated to a down-regulation of pAKT levels as demonstrated in the Mino cells. This suggests that PI3Kα inhibition also can overcome the anti-proliferative

resistance to ibrutinib conferred by the MS-5 stromal cells. Although ibrutinib only showed a transient effect on MCL tumor growth *in vivo* (see above), a significant and synergistic inhibition of tumor growth *in vivo* was observed when ibrutinib and BYL719 were administrated together. Collectively, these results show that addition of a PI3Kα inhibitor to ibrutinib can overcome (or delay development of) ibrutinib resistance in MCL.

As cell adhesion to stromal cells is required for the development of MCL cell resistance to ibrutinib, we investigated whether adhesion molecules expressed on MCL's and stromal cells may play a role in the development of ibrutinib resistance. Based on published data, the adhesion molecule VLA-4 is highly expressed on several MCL cell lines (the Cancer Cell Line Encyclopedia (CCLE, https://portals.broadinstitute.org/ccle/home). To demonstrate whether VLA-4 was involved in the development of ibrutinib resistance, Mino and MS-5 cell co-cultures were treated with a VLA-4 (CD49d) blocking antibody. We observed that the VLA-4 blocking antibody strongly reduced Mino cell adhesion to the stromal cells, suggesting that the VLA-4 indeed is involved in the attachment. Furthermore, combined treatment with ibrutinib and VLA-4 blocking antibody not only impaired the restart of MCL cell growth after drug removal, but also synergistically induced cell apoptosis compared to single drug treatment. Moreover, treatment with ibrutinib+VLA-4 blocking antibody further reduced pAKT levels when compared to ibrutinib±IgG treatment These results clearly demonstrate that stromal cell interaction with VLA-4 plays an important role in evoking the stromal cell-mediated ibrutinib resistance.

The above results clearly demonstrated that stromal cells should be considered to be an important contributor to ibrutinib resistance. They not only provide the tumor cells with a protective niche to avoid cell apoptosis, but also support tumor cell regrowth after cessation of ibrutinib treatment. Importantly, the results suggest ways to overcome stromal cell-mediated ibrutinib resistance by inhibiting either PI3Kα or blocking VLA-4 signaling.

4 CONCLUSIONS, SIGNIFICANCE AND FUTURE PERSPECTIVES

4.1 THE MECHANISMS OF ERB-MEDIATED NHL INHIBITION AND TRANSLATIONAL ASPECTS

We experimentally demonstrate that estrogens play a protective role in lymphoma progression. We also show that ERβ selective agonists inhibit lymphoma growth. Interestingly, this seems to be the case for all the lymphoma subtypes so far tested (MCL, DLBCL and BL). ERβ selective agonists not only inhibit lymphoma growth, but also impair tumor dissemination, which in MCL, at least partly, can be explained by inhibition of tumor vascularization. Considering the potent inhibition of lymphoma growth by ERβ agonists (at least as good as the newly developed targeted drug ibrutinib, data not shown), the ERβ seems to be a useful target for development of new drugs against lymphomas. The use of highly ERβ selective agonists (alone or in combination with other targeted drugs operating through other signaling pathways) should reduce side effects normally linked to the use of non-selective estrogens also interacting with ERα. Selective ERβ agonists that have been used in clinical trials to treat other diseases have shown that they are safe.

Interestingly, a majority of the lymphomas seems to express ERβ1 (>89% of primary DLBCL samples, 4 out of 4 tested primary MCL samples), the ERβ isoform capable of binding a ligand. Furthermore, identifying ERβ1 expression in individual lymphoma samples should be valuable in recognizing patients that may benefit from selective ERβ1 agonist treatment, similar to the situation for breast cancer where ERα expression will determine if the patient will undergo treatment with ERα antagonists or aromatase inhibitors. In addition establishing ERβ1 expression in the lymphoma samples may also be a useful biomarker for evaluating prognosis. To note is that this study does not consider a function for the non-ligand-binding ERβ2-5 isoforms for responsiveness or as a prognostic indicator. In fact, some studies of other cancer forms have shown that for example ERβ2 and ERβ5 may equally or better correlate to prognostic outcome.[106-108] In the case of CLL, it has been shown that high nuclear ERβ2 expression is a poor prognostic factor.[109] Thus, further investigations about the roles of the ERβ splice variants in lymphomas are necessary.

Although several experiments suggest that the growth inhibitory effect of the selective ERβ agonists are mediated via ERβ (high selectivity of DPN and especially KB9520 for ERβ binding; the ER antagonist ICI182.780 blocks the effect of selective ERβ agonists DPN;

selective ERα agonist PPT does not cause inhibition of tumor growth), definitive proof of an ERβ-mediated effect is still lacking. In order to finally establish an ERβ-mediated effect, a lymphoma cell in which ERβ expression has been ablated or knocked down is needed. Our attempts to establish such a lymphoma cell line have so far failed. Such ERβ lymphoma cell line would be useful to test in xenograft tumor mouse models where the effect of ERβ selective agonists can be compared to the response of tumors generated by control lymphoma cells in which ERβ expression has not been altered.

An additional puzzling observation which so far has not obtained a clear explanation is the clear increase in lymphoma tumor growth following ovariectomy or blocking of estrogen synthesis by aromatase inhibition in contrast to the weak inhibition of lymphoma growth following E2 administration. This might indicate that additional factors than only E2 may be involved in the regulation of lymphoma growth. Furthermore, to note is that our studies have so far only addressed the role of estrogens and ERβ in lymphoma progression. Further experimental studies are needed to address whether estrogens also affect lymphoma initiation as suggested by epidemiological studies e.g. by using mouse models which spontaneously develop lymphomas.

Additional understanding of the mechanisms and target genes involved in estrogen regulation of lymphoma growth would be obtained by performing global gene expression profiling. So far we have had problems deriving information from microarrays (Affymetrix) using tumor material from xenografts, possible due to murine tissue infiltration (cross-reacting probes) or due to a very high biological variation within the experimental groups. The prior problem might be overcome by purifying MCL tumor cells from the tumor tissue e.g. by purifying CD19 positive cells before analysis. An alternative and straighter strategy would be to analyze gene expression by RNA sequencing, which can distinguish gene expression in the human tumor cells from tumor infiltrating host murine cells. Identifying changes in the lymphoma cells of the TME (murine) might also help to evaluate the role of the TME in the lymphoma response to estrogens. This is particularly relevant as the response of human lymphoma cells in culture to estrogens is week or absent in contrast to a clear tumor growth inhibiting effect *in vivo*.

4.2 STROMAL CELL-MEDIATED IBRUTINIB RESISTANCE AND STRATEGIES TO OVERCOME THIS RESISTANCE

We have shown that attachment of MCL cells to stromal cells allows MCL cells to escape ibrutinib-induced cell apoptosis and help MCL cells to restart proliferation after ibrutinib is

removed. This may be due to dysregulated PI3Kα/AKT signaling, which we propose is activated by the interaction of the MCL expressed adhesion molecule VLA4 with its ligands VCAM1 and fibronectin. Supporting this is that inhibition of PI3Kα or blocking VLA4 will overcome stromal cell-mediated ibrutinib resistance of MCL cells. For example, the PI3Kα inhibitor BYL719 combined with ibrutinib showed a synergistic effect in inducing apoptosis of primary MCL cells and in inhibiting tumor growth *in vivo* in mice grafted with a MCL cell line.

Only in a limited number of cases of primary or acquired MCL ibrutinib resistance can the resistance be explained by mutations of the BTK, the target of ibrutinib. Thus other mechanisms for ibrutinib resistance must operate. The TME has long been considered as an important component in inducing resistance of tumor cells to cytotoxic drugs, providing a protective niche which will allow tumor cell survival and tumor relapse. The mechanism of acquired microenvironment-mediated ibrutinib resistance is not well defined and it would be a great advancement if the stromal cell-mediated ibrutinib resistance could be overcome. Although we have confirmed that VLA4-induced dysregulation of PI3K/AKT signaling is an important event for MCL cell to survive, we still are not able to exclude the possibility of other signaling pathways involved in mediating the stromal cell-mediated ibrutinib resistance. One way to get further insight into the mechanisms involved in stromal cell-mediated ibrutinib resistance would be to compare differences in gene expression of ibrutinib-treated MCL cells in the presence or absence of stromal cells. This may lead to identification of alternative targets that could be targeted in order to overcome ibrutinib resistance and more effectively prevent relapse.

However, as shown by us, detachment of MCL from stromal cells by blocking VLA4 potentiates the ibrutinib response. This supports that the MCL cell microenvironment, especially stromal cells, is a critical factor in mediating ibrutinib resistance. Thus, detaching MCL cell from the stroma by blocking adhesion molecular i.e. VLA4, would be an additive approach to be considered to overcome ibrutinib resistance. Once MCL cells are mobilized to peripheral organs or blood, they will lose adhesion molecular stimulated survival signals and regain sensitivity to ibrutinib. In summary, we suggest that combined treatment with ibrutinib and a PI3Kα inhibitor, alternatively blocking VLA-4, may be a promising therapeutic strategy to overcome stromal cell-mediated ibrutinib resistance in MCL.

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